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Nitrogen Dioxide Exposure and Lung Antioxidants in Ascorbic Acid-Deficient Guinea Pigs

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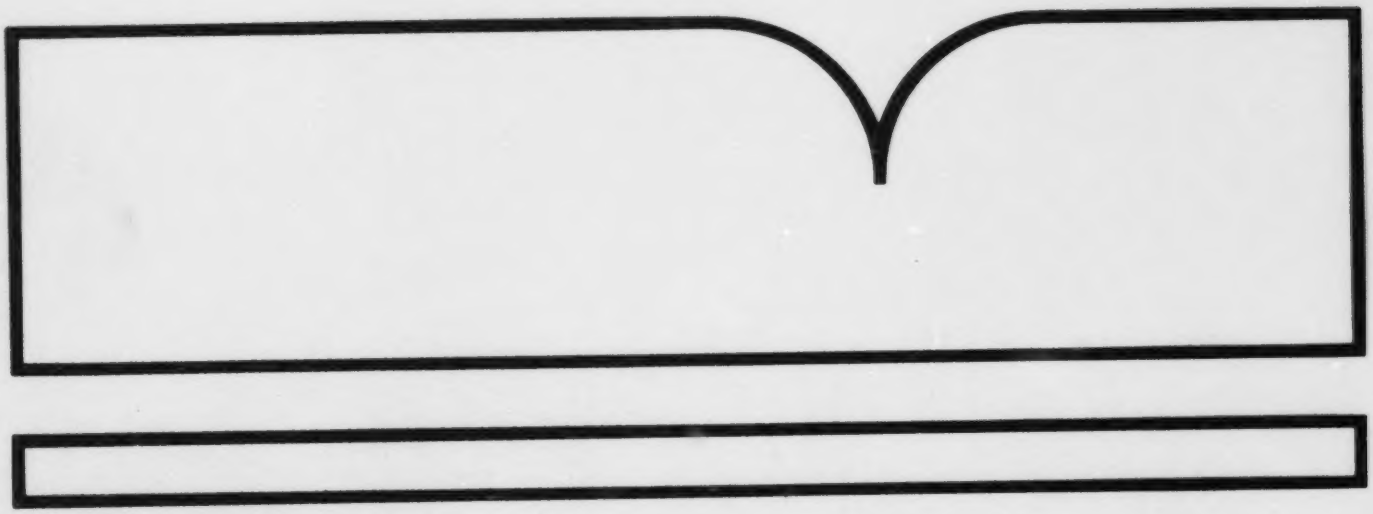

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Nitrogen Dioxide Exposure and Lung Antioxidants in Ascorbic Acid-Deficient Guinea Pigs[1]

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Nitrogen Dioxide Exposure and Lung Antioxidants in Ascorbic Acid-Deficient Guinea Pigs. HATCH, G. E., SLADE, R., SELGRADE, M. K., AND STEAD, A. G. (1986). *Toxicol. Appl. Pharmacol.* **82**, 351–359. We have previously found that ascorbic acid (AA) deficiency in guinea pigs enhances the pulmonary toxicity of nitrogen dioxide (NO_2). The present study showed that exposure to NO_2 (4.8 ppm, 3 hr) significantly increased lung lavage fluid protein (a sensitive indicator of pulmonary edema) only in guinea pigs fed rabbit chow (a diet not supplemented with vitamin C) for at least 7 days, at which time lung AA was about 50% of normal. The rabbit chow diet did not cause reduced body weight as did commercial synthetic scorbutic diets, even when they were supplemented with AA. After 14 days of feeding rabbit chow, lung AA was reduced to 15% of control. At this time, α-tocopherol (AT) in the same lungs was reduced to 85% of control, and lung nonprotein sulfhydryls (NPSH) were increased to 114% of control. Exposure of the guinea pigs to NO_2 (4.5 ppm, 16 hr) increased wet lung weight and further altered the antioxidants in deficient (but not normally fed) animals in the following manner: NPSH content was increased to 130% of control, AT was decreased to 74% of control, and AA was increased from 15 to 50% of control. These findings suggest that depletion of AA in guinea pigs removes an important defense against NO_2. The lung appears to be able to partially compensate for the dietary lack of antioxidant by accumulating AA from other tissues and by increasing NPSH concentrations. However, sufficient exposure to NO_2 leads to oxidation of AT and pulmonary edema. Conditions in which NO_2 produced edema were accompanied by only a slight consumption of AT, and no detectable oxidation of lung AA or NPSH. 1986 Academic Press, Inc.

The possibility that vitamin C deficiency could increase the pulmonary toxicity of nitrogen dioxide (NO_2) was first suggested by studies of Sherwin and Carlson (1973), who reported an increased concentration of lung lavage fluid protein in guinea pigs which had been fed rabbit chow, a feed not supplemented with this vitamin. Subsequent to this report, we assayed ascorbic acid (AA) in the blood of guinea pigs fed rabbit chow and found that these animals were indeed AA depleted and were highly susceptible to NO_2-induced elevations in lavage fluid protein and lipids (Selgrade *et al.*, 1981). Since the excess proteins found in lavage fluid had an electrophoretic profile similar to plasma, the increase in protein appeared to be associated with leakage of plasma into the air spaces. This finding was consistent with that of Alpert *et al.* (1971) who showed that ^{125}I-labeled serum albumin injected into the blood accumulates in the lung lavage fluid of animals exposed to ozone. Our previous studies also showed that guinea pigs fed a normal diet and exposed to 5 ppm NO_2 for 72 hr had no de-

[1] This report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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tectable increases in lavage fluid protein, while animals whose blood concentrations of AA were reduced to 25% of normal had sixfold elevations in lavage fluid protein and a high mortality rate. Shorter exposure times (3 hr) at lower NO_2 concentrations (3 ppm) were still effective in increasing lavage fluid protein in AA-depleted animals.

Since some interactions between diet and air pollutant toxicity, such as the effects of vitamin E depletion, require prolonged dietary restrictions that are only rarely encountered in humans, it was of interest to further characterize the interaction between vitamin C and NO_2 under conditions of mild dietary modification. Therefore, in the present study we examined the effect of shortening the time of feeding rabbit chow on lung and blood AA concentrations and on NO_2-induced lavage fluid protein accumulation. We simultaneously measured the lung concentrations of three major antioxidants in lung tissue, AA, α-tocopherol (AT), and nonprotein sulfhydryls (NPSH). In addition to characterizing the antioxidant status of the lungs in each guinea pig, it was anticipated that this approach would detect alterations in lung antioxidants under conditions where lung damage is induced.

METHODS

Animals and nonpurified diets. Young adult Hartley-strain guinea pigs (300 to 350g) were purchased from Charles River Breeding Labs (Kingston, N.Y.) and housed in temperature- and humidity-controlled rooms. Normal diet was Wayne guinea pig diet (Allied Mills, Inc., Chicago, Ill.), supplemented with 1000 ppm vitamin C. The manufacturer's published assay showed identical concentrations of 46 dietary constituents in the two feeds except for slightly higher fiber, linoleic acid, and zinc in the guinea pig chow. To make animals vitamin C deficient prior to NO_2 exposures they were fed Wayne rabbit ration (contains no vitamin C supplement) from a single milling date lot (termed "rabbit chow" hereafter). Water only was provided during exposures.

Purified diets. In two experiments to test the efficacy of purified diets, animals were fed commercial casein-based AA-deficient chow. The first study used Reid–Briggs Diet No. 901911 (ICN, Cleveland, Ohio), and the second study used Reid–Briggs diet supplemented with arginine (Reid and Mickelson, 1963; Teklad, Madison, Wisc.). In the latter study, half of the animals were given a daily oral supplement of 20 mg AA/kg body wt, as a 2% solution in 10% sucrose, made fresh weekly.

NO_2 exposures. Animals were exposed to NO_2 as previously described (Selgrade *et al.*, 1981). In the first set of exposures, groups of male guinea pigs maintained on rabbit chow for various lengths of time were exposed to 4.8 ppm NO_2 or filtered air for 3 hr. The lungs of the animals were lavaged 16 hr after the exposure ended.

A final set of NO_2 exposures included four groups of four animals (two of each sex) as follows: (1) air exposed, normal diet, (2) air exposed, rabbit chow, (3) NO_2 exposed, normal diet, and (4) NO_2 exposed, rabbit chow. There were four replicates of the exposure over an 8-day period. The animals were exposed for 16 hr (4:00 PM to 8:00 AM) either to filtered air or to 4.5 ppm NO_2. Maximum variations in chamber NO_2 concentrations never exceeded 8.0% and were usually less than 1.2% of the mean chamber concentration. Animals were randomly killed within 2 hr of the end of the exposure.

Lavage fluid preparation and assay. Animals were anesthetized by ip injection of pentobarbital (Nembutal, 50 mg/kg, diluted 1:5 in saline) and exsanguinated by severing the abdominal aorta. The diaphragm was cut to collapse the lungs, and the trachea was exposed. A blunt metal needle (Popper and Sons, New York, N.Y.) was inserted into the exposed trachea, secured with a ligature, and a volume of saline warmed to 37°C and equal to 35 ml/kg body wt was injected. This amount of saline was equivalent to about 90% of the total lung capacity based on allometric data published by Takezawa *et al.* (1980). The injected saline was immediately withdrawn until resistance was felt, and the same fluid was infused and withdrawn two additional times. Lavage fluid samples were placed on ice until centrifuged to remove cells (400*g*, 15 min). Protein concentrations were determined in the supernates by the method of Lowry *et al.* (1951).

Lung tissue preparation. Animals were anesthetized with halothane (Halocarbon Laboratories, Hackensack, N.J.), blood samples taken by cardiac puncture, and then the abdominal aorta was severed. Lung samples were removed and homogenized using a Polytron tissue homogenizer (Brinkman Instrument Co., Westbury, N.Y.) equipped with an anaerobic probe generator. Tissue samples (0.1 to 0.2 g) for AA and NPSH analyses were homogenized in 3.0 ml of 6.0% (w/v) metaphosphoric acid and centrifuged at 20,000*g* for 20 min. Tissues for analysis of AT (~0.5 g) were homogenized in 10.0 ml of ethanol plus 2.5 ml of 25% (w/v) AA. Lung lavage fluid samples for AA analysis were centrifuged (400*g*, 15 min), the supernatant fractions were adjusted to 6% metaphosphoric acid and centrifuged at 20,000*g* for 20 min. Blood samples assayed for AA were adjusted to 6% metaphosphoric acid, mixed, and centrifuged (20,000*g*, 20 min).

Determination of ascorbic acid, nonprotein sulfhydryl, and α-tocopherol. Lung AA was determined both by a modification of the dinitrophenylhydrazine (DNPH) method of Roe and Kuether (1943) and by high-performance liquid chromatography (HPLC). In the DNPH as-

say for total AA, approximately 1.0 ml of the metaphosphoric acid supernatant fraction was oxidized with Br_2 gas, and then a 0.6-ml aliquot was added to 0.2 ml of a mixture containing 2.0% (w/v) DNPH, and 0.25% (w/v) thiourea in 9.0 N H_2SO_4. The tubes were incubated at 25°C for 24 hr (Skoza *et al.*, 1983), then 1.0 ml of ice-cold 65% H_2SO_4 was added, incubated at 25°C for 30 min, and the absorbance at 525 nm was determined.

The HPLC assay for reduced AA employed a Waters radial compression Z-module with a Radial-PAK μM Bondapak C_{18} column cartridge (Waters Associates, Milford, Mass.). Metaphosphoric acid homogenate supernatant fractions were filtered (0.45-μm) and injected directly onto the column in 20-μl volumes. The mobile phase was 0.3 M β-alanine plus 0.5% (w/v) dicyclohexylamine phosphate, adjusted to pH 3.5 with orthophosphoric acid. The flow rate was 1.5 ml/min, and AA was detected by its absorbance at 254 nm. The retention time of the AA was 4.5 min.

NPSH in homogenate supernatant fractions was determined by the method of Sedlak and Lindsay (1968). Results using metaphosphoric acid as protein precipitant did not differ from those obtained using trichloroacetic acid.

Ethanol homogenates for analysis of AT were saponified by addition of 1.0 ml of 10 N KOH under N_2 and incubation at 45°C for 20 min. Tubes were chilled on ice, then 10.0 ml of ice-cold hexane containing 1.25 mg% BHT was added, mixed for 1 min, and centrifuged (1000 rpm, 2 min). An 8.0-ml sample of the hexane layer was removed, dried under N_2, and redissolved in 0.5 ml of absolute methanol. The methanol extract (20 μl) was injected directly into the HPLC column (Waters Z-module C_{18} cartridge) according to the method of DeLeenheer *et al.* (1979). A 100% methanol mobile phase with a flow rate of 2.0 ml/min and UV (292 nm) detection were employed. Rabbit chow and guinea pig chow were prepared by grinding 0.5 g samples with a mortar and pestle and were assayed the same as for lung samples.

Statistical methods. In studying the effects of the length of time on the rabbit chow diet on lavage fluid protein and AA concentrations, Williams' (1972) test was applied. This procedure assumes a monotonically increasing (or decreasing) response with time.

For data arising from the 16 hr NO_2 exposure, a three-way analysis of variance (ANOVA) model, with replicate as a block, was employed to test for the influence of sex, diet, NO_2 exposure, and their interactions on lung antioxidant concentrations and on lung and body weights. When necessary, the response was log transformed to satisfy the homogeneity of variance assumption implicit to ANOVA. Contrasts between group means were tested by using ANOVA variance estimates to form the appropriate *t* statistic.

RESULTS

Comparison of synthetic diets and rabbit chow. Table 1 shows the results of two experiments in which guinea pigs were fed either Wayne guinea pig chow, rabbit chow, or a synthetic diet. In the first experiment, guinea pigs fed the normal chow for 9 days showed a 6% increase in body weight while those fed the Reid–Briggs synthetic diet lost 10 to 14% of their body weight, regardless of whether the diet was supplemented with AA. In the second experiment, animals fed both the Wayne guinea pig and rabbit chows showed a weight gain of 24 to 27% after 14 days, while those groups fed the synthetic diet showed only a 0 to 5% weight gain regardless of whether or not they were given a daily po AA supplement. Three of the animals in the second experiment showed a greater weight loss than the others from the outset of the experiment, and all three died between Days 10 and 16. These results convinced us that feeding purified diets was not an effective way of studying acute AA deficiency in adult guinea pigs.

TABLE 1

GROWTH OF GUINEA PIGS ON DIFFERENT DIETS

	n	% Change in body wt ± SE
1—9 days on diet[a]		
Wayne guinea pig chow	15	+5.87 ± 0.98
Reid–Briggs diet, +AA	14	−9.97 ± 3.30
Reid–Briggs, diet, −AA	14	−13.90 ± 2.67
2—17 days on diet[b]		
Wayne guinea pig chow	5	+26.6 ± 1.8
Wayne rabbit chow	5	+24.2 ± 1.0
Reid–Briggs diet (+Arg) + AA	3[c]	−0.6 ± 3.3
Reid–Briggs diet (+Arg) −AA	5[d]	−4.9 ± 9.0

[a] Guinea pigs were fed diets *ad libitum;* supplemented Reid–Briggs diet contained 1 g AA/kg.

[b] Guinea pigs were fed diets *ad libitum;* AA supplement (2 mg/kg body wt) was given orally each day.

[c] Two animals died between Days 10 and 13.

[d] One animal in this group died on Day 16.

Effects of time of rabbit chow feeding on NO_2-induced excess lung lavage fluid protein. Four experiments were performed in which guinea pigs were maintained on the same lot of rabbit chow for varying times up to 17 days. At the end of each period on the diet, both deficient and normal animals were exposed to 4.8 ppm NO_2 for 3 hr and their lungs were

lavaged 16 to 18 hr later. A plot of the lavage fluid protein concentration versus days on rabbit chow is shown in Fig. 1A. Seven days was the shortest time of feeding rabbit chow that resulted in elevated lavage fluid protein following exposure when analyzed by the method of Williams (1972). Further increases in the time of feeding rabbit chow caused proportionally greater increases in the NO_2 effect. At 17 days on the diet, lavage fluid protein was almost three times that observed in control animals.

Effects of time of rabbit chow feeding on lung ascorbic acid levels. In three of the four experiments in which lavage fluid protein was examined, lung, blood, and lavage fluid AA concentrations were also determined. A decreasing trend was seen in the lung AA content with increasing time on the rabbit chow diet (Fig. 1B). The Williams test on pooled data of the three experiments indicated a significant reduction of lung AA of approximately 50% after 4 days of feeding rabbit chow. Further reduction in AA occurred until it fell to 10% of control after 17 days.

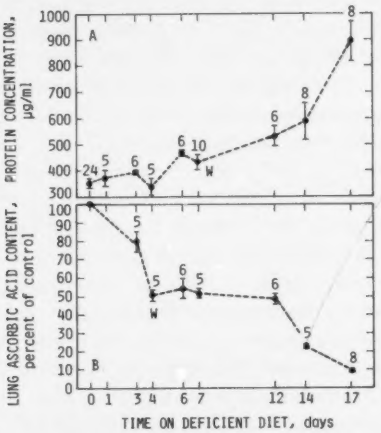

FIG. 1. Effects of time of feeding rabbit chow on NO_2-induced lavage fluid protein accumulation and lung ascorbic acid concentrations. Male guinea pigs were maintained on rabbit chow for 17 days. (A) Animals were exposed to 4.8 ppm NO_2 or filtered air for 3 hr at various times up to 17 days on the diet and their lungs lavaged 16 hr after exposures. (B) Lung ascorbic acid was determined at various times up to 17 days. $\bar{x} \pm$ SE are given for n animals as indicated at each data point. Values marked with W indicate the shortest time producing a significant effect (Williams test of raw data).

Concentrations of AA in whole blood and lung lavage fluid also appeared to decrease with time on the diet. However, greater variability was noted in these values (data not shown). Attempts to relate the lavage fluid protein concentration to the AA concentration of lung, blood, or lung lavage fluid on an animal-by-animal basis failed to reveal a more clear-cut relationship between these measurements than was possible by comparing means from groups of animals.

Effects of 16-hr NO_2 exposure on guinea pigs fed rabbit chow. The possibility that AA, as well as NPSH and AT, might be altered by the NO_2 exposure itself was investigated in the following experiment which was designed to combine both a high level of AA deficiency and NO_2 exposure. To detect possible interactions between AA, NPSH, and AT, all three antioxidants were determined in each animal lung. The NO_2 exposure was lengthened to 16 hr, a time which we had found to cause not only severe lavage fluid protein accumulation (>1500 µg/ml), but also elevated lung wet weight in AA-deficient animals. Lavage fluid protein was not determined because the lung wet weight served as an adequate indicator of edema, and because the lungs could be processed more rapidly following exposure when no lavage was performed. A series of four experiments was performed, each consisting of 16 animals composed of equal numbers of both males and females.

Table 2 shows a summary of the overall statistical analysis comparing male and female animals. No significant effects due to exposure or diet were seen on body weight, but there was a significant ($p < 0.001$) effect attributable to the sex of the animals: females were about 10% lighter than males of the same age. An interaction between diet and the sex of the animals was also observed ($p = 0.02$). This interaction was apparently due to the different effect of the diets on each sex. Rabbit chow-

TABLE 2

EFFECTS OF DIET AND SEX ON BODY WEIGHT AND LUNG α-TOCOPHEROL[a]

	Body wt (g)	α-Tocopherol (μg/lung)
Male—GP-Ch	396.6 ± 13.2	51.4 ± 3.3
Male—R-Ch	411.9 ± 13.2	41.5 ± 3.4
Female—GP-Ch	372.8 ± 4.5	47.7 ± 3.9
Female—R-Ch	351.3 ± 6.4	36.1 ± 2.4

ANOVA Results

Factor	Probability	Probability
Diet	0.610	0.001
Sex	<0.001	0.015
Diet × Sex	0.020	0.522

[a] Animals were fed either normal guinea pig chow (GP-Ch) or rabbit chow (R-Ch) for 14 days prior to exposure to 4.5 ppm NO_2 for 16 hr. All data are $\bar{x} \pm$ SE, $n = 16$ animals per group.

fed males were slightly (4%) heavier than males fed a normal diet, while rabbit chow-fed females were 6% lighter than normally fed females. Lung AT content showed significant effects of both diet ($p < 0.001$) and sex ($p = 0.015$). Females tended to have lower AT concentrations than males, and the rabbit chow diet lowered these values about equally (20 to 25%) in each sex. When the data were pooled across exposure and diet groups, males had about 12% higher AT concentrations than females.

Since there were no sex-related interactions in other measurements, the data from males and females were subsequently pooled for analysis. Lung wet weights in guinea pigs fed rabbit chow and exposed to NO_2 were significantly elevated about 20% above the other three groups (Table 3). Because of this edemagenic effect, AA, AT, and NPSH were expressed on a per lung basis rather than as a concentration in the lung. The edema itself would have made the antioxidant concentrations appear lower because plasma (which presumably increases the lung weight in edema) contains far less of these antioxidants than does lung tissue.

Two techniques were used to analyze lung AA content: a HPLC assay which measured only reduced AA and a colorimetric assay which measured total AA (oxidized + reduced). Essentially the same pattern of effects were seen with both methods, although the amounts determined by HPLC were 25 to 60% lower. ANOVA showed significant effects of diet and NO_2 exposure (Table 3). Lung AA content was reduced to about 15% of normal by the rabbit chow diet but increased to 50% of normal when the deficient animals were exposed to NO_2 (comparison 3 vs 4). No significant effect of NO_2 was seen in animals fed the normal diet (1 vs 2).

Lung AT was significantly lowered (by 15%) by the rabbit chow diet (comparison 1 vs 3). NO_2 exposure further lowered the AT concentrations another 11% in the animals fed rabbit chow (3 vs 4), but it had no effect in animals fed normal chow.

The rabbit chow diet alone significantly increased lung NPSH content by about 14% in air-exposed animals (1 vs 3, Table 3), and NO_2 exposure further increased NPSH by another 16% (3 vs 4). The NO_2-induced increase in NPSH was apparent only in the guinea pigs which were fed rabbit chow.

DISCUSSION

The present study examined the approximate degree of AA deficiency required to detect NO_2-induced increases in lavage fluid protein in guinea pigs, and the effects of NO_2 exposure on the lung content of AA, NPSH, and AT in AA-depleted and normal animals.

Early in these studies we found that synthetic casein-based diets were not accepted well by adult guinea pigs. Although the addition of arginine to the synthetic diets had been reported to improve weight gains (Reid and Mickelsen, 1963), our trials with arginine-supplemented Reid–Briggs diet still yielded lower than normal weight gains (Table 1). Our lack of success with the synthetic diets might be explained by the fact that in the published

TABLE 3

NO_2 EFFECTS ON LUNG ANTIOXIDANTS IN NORMAL AND VITAMIN C-DEFICIENT GUINEA PIGS[a]
$\bar{x} \pm$ SE FROM TISSUE MEASUREMENTS

	Lung wt (g)	Reduced ascorbic acid[b] (μg/lung)	Total ascorbic acid[c] (μg/lung)	α-Tocopherol (μg/lung)	NPSH (mg/lung)
Diet–exposure					
1. GP-Ch-Air	2.19 ± 0.05	255.2 ± 26.2	493.6 ± 34.8	49.7 ± 3.1	1.39 ± 0.05
2. GP-Ch-NO_2	2.19 ± 0.05	412.4 ± 92.4	554.2 ± 70.2	49.3 ± 3.2	1.37 ± 0.06
3. R-Ch-Air	2.27 ± 0.05	32.8 ± 6.1	79.5 ± 11.7	41.6 ± 3.1	1.59 ± 0.06
4. R-Ch-NO_2	2.72 ± 0.12	115.2 ± 33.9	236.5 ± 68.5	36.1 ± 2.8	1.84 ± 0.09
Effect		Significance probabilities from ANOVA *F* tests			
Gas (NO_2)	0.0019	0.0024	0.0371	0.0619	0.0767
Diet	0.0001	0.0001	0.0001	0.0001	0.0001
Gas × Diet	0.0014	0.0802	0.0789	0.1020	0.0275
Sex	0.8650	0.0993	0.4320	0.0134	0.2223
Contrast		Significance probabilities from *t* tests of specified contrasts[d]			
1 vs 2	0.9495	0.3264	0.8074	0.8643	0.7458
3 vs 4	0.0001	0.0009	0.0075	0.0145	0.0057
1 vs 3	0.2976	0.0001	0.0001	0.0011	0.0094

[a] Guinea pigs were fed either normal chow (GP-Ch) or rabbit chow (R-Ch) for 14 days, then exposed to 4.5 ppm NO_2 for 16 hr. Lungs were assayed immediately after exposure. $n = 16$ animals for each of the four groups (56 degrees of freedom for error in the ANOVA). Data are $\bar{x} \pm$ SE from tissue measurements.

[b] Assay of reduced ascorbic acid by HPLC method.

[c] Assay of total (reduced plus oxidized) AA by DNPH method.

[d] Two-sided tests of difference between group means by variance estimates from the ANOVA.

studies, the guinea pigs were started on the synthetic diets earlier in life (Reid and Briggs, 1953). Also, maximum weight gain was not claimed by these investigators: only satisfactory weight gain. Since we were interested in producing relatively mild vitamin C deficiency states, which have been achieved previously using rabbit chow (Selgrade *et al.*, 1981), we used this diet in the present study and attempted to define the degree of deficiency by assay of AA and AT in the lungs of the guinea pigs. Interesting results were obtained; however, further work is needed to improve methods for controlling dietary antioxidants in guinea pigs.

Our previous studies showed that when blood AA concentrations were reduced to 25% of normal by feeding rabbit chow for 2 to 3 weeks there was an enhanced susceptibility to NO_2, as measured by lavage fluid protein accumulation (Selgrade *et al.*, 1981). In the present study, increases in lavage fluid protein following exposure to NO_2 (5 ppm, 3 hr) were significant after 7 days on rabbit chow, at which time there was approximately a 50% reduction in lung AA concentrations. It is probable that higher concentrations of NO_2 or longer exposure times would require smaller reductions in AA to produce significant effects. The assay of lavage fluid protein provides data that appear indicative of low-level pulmonary edema. Previous studies have shown that radiolabeled albumin or other small molecules injected into the bloodstream of rats exposed to edemagenic gases leak into the pulmonary airways and are recoverable in lavage fluid (Alpert *et al.*, 1971; Valentini *et al.*, 1983). Significant accumulation of radiolabel or protein in the airways is observed at concentrations of irritant gas below those required to produce histological evidence of edema or increased wet lung weight. The majority of the

accumulated proteins in lavage fluid appear to be derived from plasma, as evidenced by the similarity of the banding patterns observed following polyacrylamide gel electrophoresis of plasma and lavage fluid from exposed animals (Selgrade *et al.*, 1981).

The combination of marked AA deficiency and a longer NO_2 exposure (16 hr) produced a frank pulmonary edema detectable as increased wet lung weight. Assay of AA, NPSH, and AT in the same lungs under these conditions revealed some interesting interrelationships between these antioxidants. Although AA depletion appeared to be the cause of increased susceptibility to NO_2, some ability to compensate by increasing AA (probably at the expense of other tissues), and a marked ability to elevate NPSH was apparent in animals which had been fed the deficient diet and exposed to NO_2. AT, on the other hand, was decreased both by the deficient diet and by the NO_2 exposure. Assay of the lungs of guinea pigs which were fed rabbit chow for 2 weeks showed a definite depletion of AA. However, other antioxidants were also affected: AT was slightly lowered while NPSH was increased. Other investigators have reported lower AT in vitamin C-deficient animals (Kanazawa *et al.*, 1981; Hrubá *et al.*, 1982). Our assay of the rabbit and guinea pig chows indicated essentially no difference (39.8 and 41.6 μg/g, respectively) in AT concentrations between these two diets; however, better control of dietary formulations would be necessary to conclude that AA deficiency might enhance AT deficiency. Further studies are needed which assay all antioxidants in the same tissue sample. It has been postulated that AA, AT, and NPSH are interrelated in providing protection against oxidant-induced damage (Tappel, 1962; Menzel, 1970), and *in vitro* as well as *in vivo* interactions between AA and AT and between AA and NPSH have been demonstrated during oxidant challenge (Packer *et al.*, 1979; Reddy *et al.*, 1982; Barclay *et al.*, 1983; Leung *et al.*, 1981).

The toxic effects of NO_2 which were detectable in animals fed rabbit chow were probably more a result of the 85% lower AA than the 10% lower AT content of the lungs. Studies in which AT levels have been measured following dietary restriction suggest that alterations of AT need to be more severe in order to cause differences in sensitivity to NO_2 (Sevanian *et al.*, 1982; Elsayed and Mustafa, 1982). It was apparent that AT was lowered under conditions in which pulmonary edema was present (Table 3, group 4). No such disappearance of AA or NPSH was detected under the same conditions of NO_2 exposure. In fact, these concentrations were increased relative to those of unexposed animals. Ascorbate accumulation in the lungs following exposure to a similar oxidant gas, ozone, has been observed in mice (Dubick *et al.*, 1983). Other studies which used more severe exposure conditions (45 ppm NO_2, 10 hr) than those used here showed decreased lung AA (Leung and Morrow, 1981). Mechanisms have been identified both for the active transport of ascorbate into lung cells (Wright *et al.*, 1981; Castranova *et al.*, 1983) and for maintaining ascorbate in its reduced form (Diliberto *et al.*, 1982; Leung and Morrow, 1981). Dubick and coworkers (1983) suggested that AA accumulation in the lungs may be the result of cellular influx and proliferation during repair of the gas-induced lesion. Thus, it appears that several phenomena could be happening at once, and further work will be required to clarify the responses and determine their concentration–response relationships.

We attempted to obtain an index of the redox state of AA in the lungs by using two different assays. We found that the HPLC-determined reduced-AA concentrations were always lower than the colorimetrically determined total ascorbate. This difference between HPLC and spectrophotometric assays has already been noted by Tsao and Salimi (1981), and the reason for its existence is not yet known. It may be due to differences in specificity of the two assays. The similarity in pattern of response we observed between the HPLC and colorimetric assays suggests that NO_2 and the rabbit chow diet were not af-

fecting the state of reduction of the ascorbate. Although NPSH was increased in the lungs of the deficient animals the edemagenic response to NO_2 still occurred, suggesting that NPSH did not protect against the NO_2. It is, of course, possible that the NPSH may have been depleted early in the exposure while the actual damage was occurring. Increased lung NPSH has been reported following NO_2, and this response appears to be a common one with oxidant gas exposure (Ospital *et al.*, 1981).

Further studies are needed to evaluate the possible significance for humans with regard to the effect of NO_2 exposure on vitamin C-deficient guinea pigs. We have measured human lung AA concentrations using samples taken from apparently normal regions of lungs from cancer surgery patients and found AA concentrations as low as or lower than the 50% depleted concentrations observed here in guinea pigs (Slade *et al.*, 1985). Guinea pigs also apparently become vitamin C-depleted more rapidly than humans. They showed lung AA concentrations that were lowered by 50% after 1 week of eating a deficient diet, while previous studies in humans required 4 to 6 weeks of a deficient diet to lower leukocyte concentrations by the same amount (Vitamin C Subcommittee, 1953). Guinea pigs have a more rapid turnover of AA than humans (Rucker and Dubick, 1981). Guinea pig chow also contains far higher amounts of AA supplementation (1 g/kg) than a normal human diet would contain.

In summary, we have investigated the role that AA plays in defending the lungs from oxidant attack by NO_2. This vitamin appeared to be a major factor in the defense because its concentrations were altered the most under conditions of increased toxic effect of NO_2. Of the antioxidants studied, only AT appeared to be significantly lowered during the time of NO_2 exposure which resulted in lung damage. AT was decreased by both the rabbit chow diet and by the NO_2 exposure. NPSH was proportionally increased in the same groups that had decreased AT. On the other hand, lung AA was lowered by the deficient diet, but NO_2 exposure elevated these values back toward normal. In spite of the apparently favorable alterations in the lung AA and NPSH brought on by NO_2 exposure, the lungs of deficient animals were damaged, as indicated either by increased wet lung weight or by increased lavage fluid protein concentrations.

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